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Reciprocal regulation of the human sterol regulatory element binding protein (SREBP)-1a promoter by Sp1 and EGR-1 transcription factors

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Abstract **Sterol regulatory element binding protein (SREBP)-1a is a transcription factor that is highly expressed in actively growing cells, and is involved in the biosynthesis of cholesterol, fatty acids and phospholipids. We have mapped the minimal human SREBP-1a promoter region to 75 bp upstream of the translation start site where we discovered a functional role for the 3 GC-boxes containing overlapping sites for the Sp1 and EGR-1 transcription factors. Intact SP1-binding sites are essential for promoter activity, whereas EGR-1 suppresses the transcription of the human SREBP-1a promoter. These results reveal a novel physiologically relevant transcriptional mechanism for the reciprocal regulation of the SREBP-1a expression.**

Keywords: SREBP-1a; Egr-1; Sp1; Gene expression; Promoter; Cell line

1. Introduction

Sterol regulatory element binding proteins (SREBPs) are members of the basic helix–loop–helix family of transcription factors that directly activate the expression of those genes involved in cholesterol and fatty acid metabolism [1]. The SREBP-1 gene encodes two almost identical proteins, SREBP-1a and SREBP-1c, which are expressed from overlapping mRNAs. A separate gene encodes a third isoform, SREBP-2 [2]. SREBP-1a is the predominant isoform in most cultured cell lines, spleen and intestine, whereas SREBP-1c predominates in the liver and in most other tissues, such as muscle and adipose tissue [3]. These data indicate that those promoters that control SREBP-1 mRNAs initiation not only respond independently to organ-specific factors, but also to metabolic stresses.

The responsible promoter regions associated with the basal transcription of the mouse SREBP-1a promoter have been identified. The promoter for mouse SREBP-1a is contained in a very small promoter-proximal region encompassing two binding sites for transcription factor SP1 that are essential for promoter activity. These two SP1 sites reside in a region that is highly conserved within the human gene, emphasizing these important *cis*-acting DNA elements [4]. In many promoters, SP1 sites overlap with binding sites for the early growth response 1 protein (EGR-1). This transcriptional factor, also known as Zif268, NGFI-A, Krox24 or TIS8, is able to either activate or repress the transcription of gene promoters at their GC-rich elements [5]. Moreover, the early growth response gene 1 (*egr-1*) expression augments the SP1 activation of non-overlapping SP1+EGR-1 sites, but inhibits SP1 activity when sites overlap by competing with SP1 for the binding site [6].

The lack of detailed experimental data in the human SREBP-1a promoter prompted us to not only investigate the responsible regions in the 5′ flanking region of the human SREBP-1a promoter, but also the transcription factors associated with the human SREBP-1a transcript expression. We mapped the basal promoter to 75 bp upstream of the ATG start codon and demonstrated a functional role for three GC-boxes containing overlapping EGR-1 and SP1 binding sites. Therefore, both these factors are implicated in the regulation of the human SREBP-1a promoter.

2. Materials and methods

2.1. Construction of the human SREBP-1a-lucifersase promoter plasmids

Two fragments containing 1545 bp and 1202 bp, corresponding to the 5′ upstream region of the human SREBP-gene, were amplified by PCR and cloned into pGEMTeasy vector (Promega, Madison, USA) to construct the pSPro-easy and pPro-easy vectors, respectively. The −2553, −1648, −1008, −889, −717, −360 and −75/+194luc plasmids were obtained by releasing restriction fragments from the pSPro-easy and pPro-easy constructs followed by subcloning into the pGL3-basic vector (Promega). Mutagenesis was performed by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), where pPro-easy was deployed as a template. All plasmids were confirmed by nucleotide sequencing. Vectors pcDNA3-Egr1 (Addgene plasmid 11729), pEBS1[4]*luc*, pCMV-Sp1 (Addgene plasmid 12097) and pAld-GCB[4] were previously described [5,7].

2.2. Cell culture and luciferase assay

Human embryonic kidney 293T cells (HEK293T cells) were cultured in DMEM containing 25 mmol/l glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin supplemented with 10% foetal bovine serum.

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Abbreviations: ChIP, chromatin immunoprecipitation; *egr*-1, early growth response gene 1; EGR-1, the early growth response 1 protein; EMSA, electrophoretic mobility-shift assay; HEK293T cells, human embryonic kidney 293T cells; NFκB, nuclear factor kappa B; SREBP, sterol regulatory element binding protein

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Cells were seeded in 6-well culture dishes and co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA. USA) with 850 ng of SREBP-1a reporter plasmids and 50 ng of a Renilla luciferase construct (pRL-TK) as an internal control. In some experiments, cells were co-transfected with 200 ng of luciferase vectors, 100 ng of pcDNA3-Egr1 or pCMV-Sp1 expression vector and 20 ng of pRL-TK. Transactivation activities were measured 24 h after transfection in a Wallac 1420 VICTOR luminometer according to the technical manual of the Dual-Luciferase Reporter Assay System (Promega).

In the RNAi experiments, HEK293T cells were co-transfected with 100 nM of synthetic pre-designed short interfering RNA (siRNA) against human *egr-1* (siRNA ID#: 146223), human *sp1* (siRNA ID# 116547 and # 143158) or silencer negative control siRNA (Ambion, Austin, TX), with 800 ng of −75/+194luc plasmid and 40 ng of pRL-TK using Lipofectamine 2000 in Opti-MEM® 1 medium (Invitrogen) according to the manufacturer's instructions. After 48 h of incubation, luciferase activities were determined as described above. The *Egr-1*, *Sp-1* and *SREBP-1* levels were monitored by RT q-PCR using the second derivative comparative C_t method. The primer sets and Taqman probes used to analyze cDNA were proprietary to Applied Biosystems (Assays-on-demand gene expression product; Applied Biosystems Foster City, CA, USA).

2.3. RT-PCR

Total RNA was isolated from HeLa, HEK293T or HepG2 cells using Trizol reagent (Invitrogen). First strand cDNA was synthesized from 1 μg of total RNA using random hexamer and expand reverse transcriptase (Roche Diagnostics, Manheim, Germany). cDNA was used as a template for conventional PCR, using specific primers of human SREBP-1 isoforms (sequence upon request). The GAPDH expression was measured as an endogenous control.

2.4. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from the HEK293T cells transfected with pcDNA3-*egr1* were prepared as previously described [8]. EMSA were performed using double-stranded DNA oligonucleotides that were end-labelled with [γ-^{32}P]ATP as described in [9]. Binding reactions were carried out for 20 min at room temperature using recombinant human SP1 protein (Promega), human EGR-1 protein synthesized from pcDNA3 expression vectors using the TNT T7-coupled reticulocyte lysate system (Promega) or nuclear extracts, 9 fmol of probe, 2 μg dI/dC in the binding buffer and 6 μg BSA. Supershifts were obtained by adding specific antibodies to the reaction mix for 1 h on ice before adding the probe. For the competition experiments, a 25-fold excess of unlabelled oligonucleotide was added to the reaction mixture. DNA–protein complexes were resolved on 6% (w/v) non-denaturing polyacrylamide gels in 0.5× TBE buffer (1× TBE is 90 mM Tris, 90 mM boric acid and 1 mM EDTA).

Protein analysis was carried out by Western blot as previously described [10] using antibodies to EGR-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.5. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) and RNApol-ChIP procedures, using isolated nuclei from formaldehyde cross-linked HEK293T cells, were performed according to Sandoval et al. [11]. Briefly, isolated nuclei from formaldehyde cross-linked cell cultures were lysed and chromatin was sonicated in a Vibra-Cell VCX-500 sonicator to yield

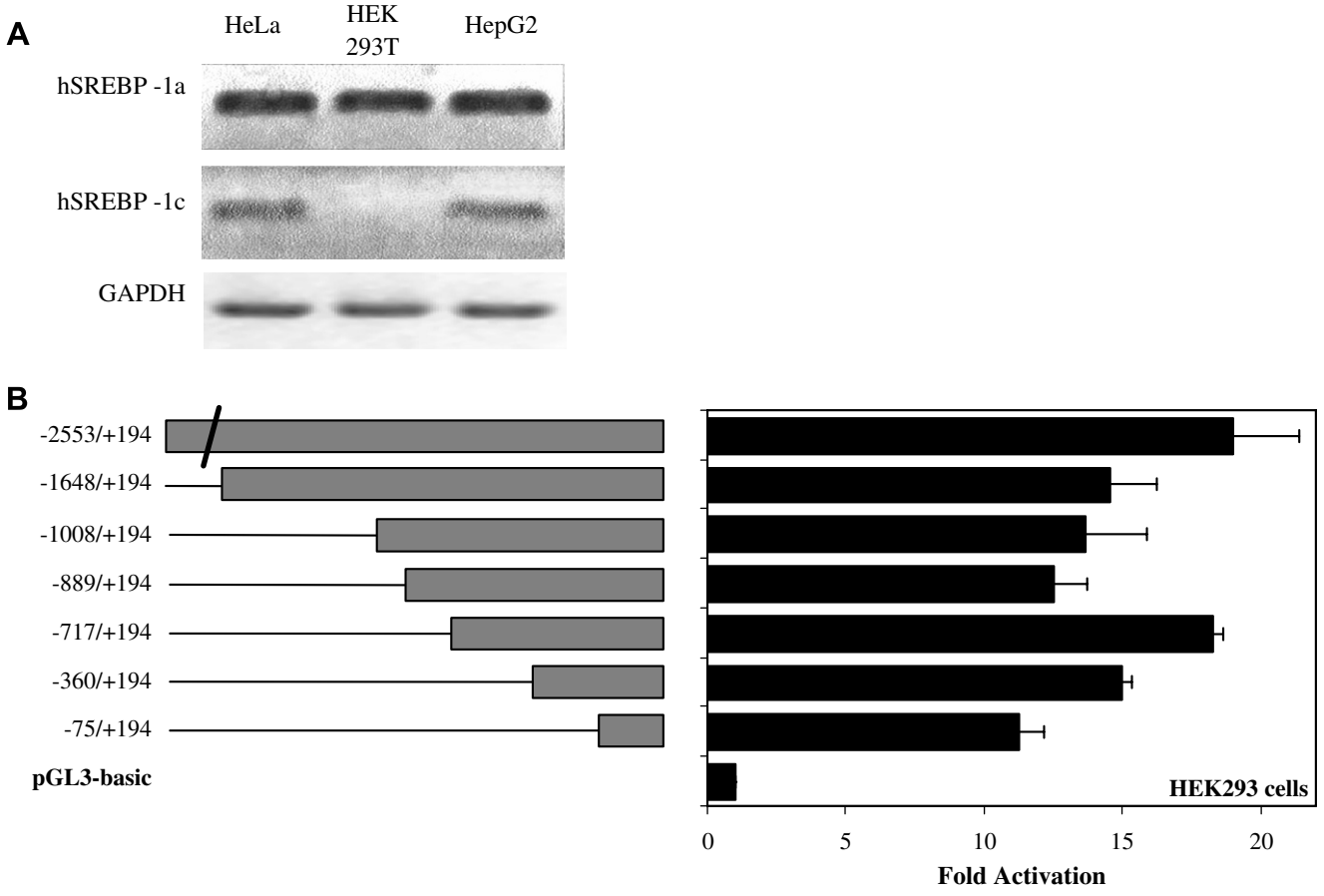

Fig. 1. Characterization of the early promoter region of the human SREBP-1a gene. (A) Total RNA was purified from different cell lines and subjected to RT-PCR. The expression of SREBP-1c was barely detectable in HEK293T cells. (B) The sequences in the SREBP-1a promoter, shown on the left-hand side, were ligated into the multiple cloning regions of the pGL3-basic vector and transfected into HEK293T cells. The fold induction relative to pGL3-basic vector (±S.E.M.), and normalized to renilla luciferase data, was calculated. The results shown are the average of four transfections run in duplicate.

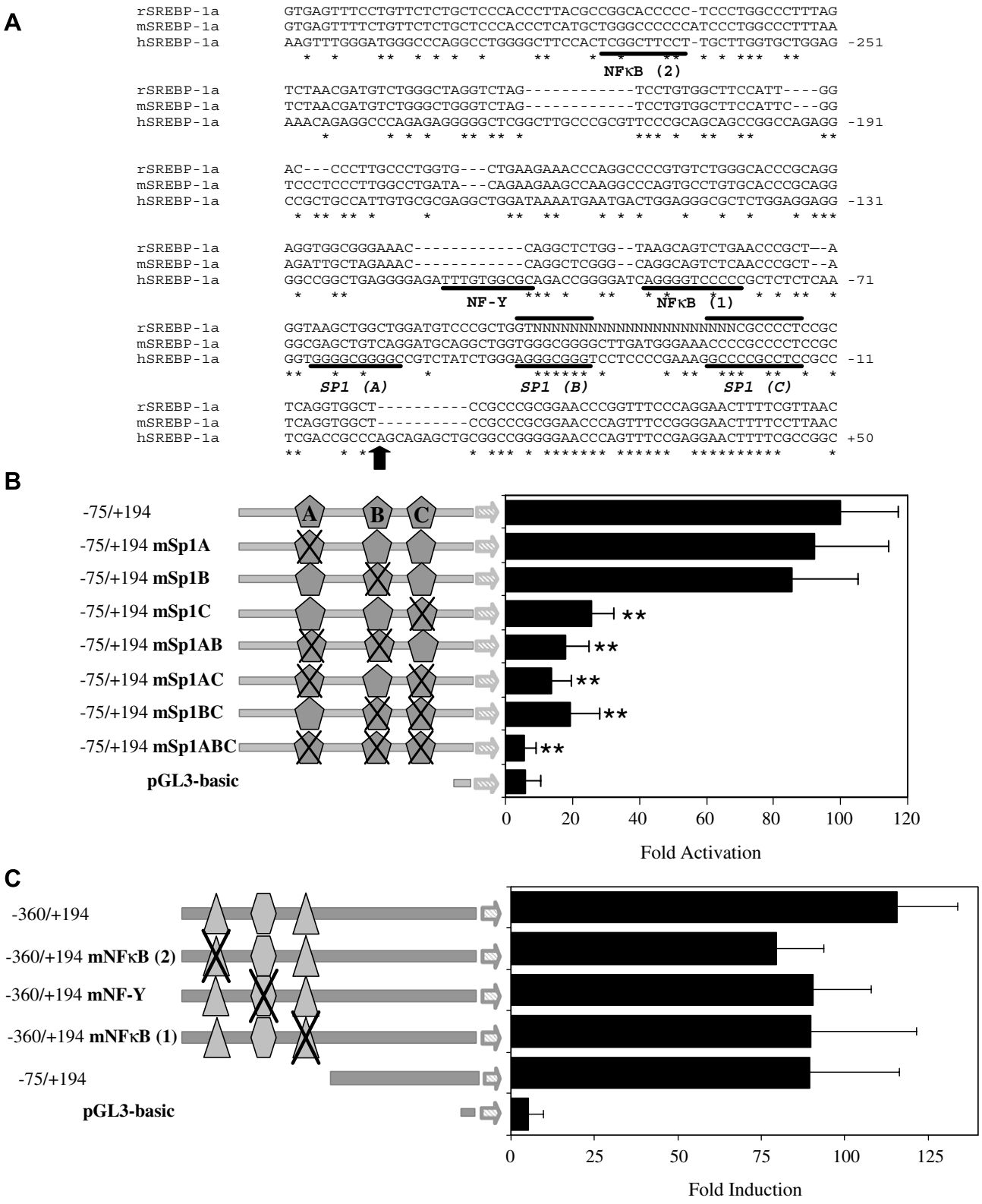

Fig. 2. SP1 binding sites are critical for the human SREBP-1a promoter activity. (A) A partial sequence alignment of the rat, mouse and human SREBP-1a promoters: the putative SP1, NF-Y and NFκB binding sites are underlined. The human SREBP-1a mRNA initiation site is denoted by an arrow. (B) Reporter gene analysis following the transfection of HEK293T cells with wild-type or indicated SP1 binding sites mutants. The data are represented as the mean firefly/renilla luciferase ratio relative to the activity of the p −75/+194luc construct (set at 100%). Each point is the means ± S.E.M. of four assays runs in duplicate. $^{**}P < 0.01$. (C) HEK293T cells were transiently transfected with p −360/+194luc plasmid or site-specific mutants, and were analyzed as above.

soluble fragments of ~500 bp. Pre-cleared chromatin was incubated with 2 μg of the corresponding antibodies against SP1 (sc-59), EGR-1 (sc-110) and RNA pol II (sc-899) (Santa Cruz). Immunoselected chromatin was eluted and formaldehyde cross-linking was reverted. The DNA from all samples was purified with a PCR purification kit (Qiagen) and used for PCR analysis. The PCR primers used to detect target sequences were as follows: *SREBP-1a* (promoter), 5′-ACTCGGCTTCCTTGCTTGGTGCTG-3′ and 5′-CGCCGGCGAAAAGTTCCTCGGA-3′; *SREBP-1a* (coding region), 5′-AGCTTACAGCACAGAACTCCCCTG-3′ and 5′-CCCCTGTGGAGCACATGGTG-3′; *a-actin* (promoter), 5-TCCAGGTCTAGCCAGTCCTG-3′ and 5′-AAAGCTGAGCCACGTCGACC-3′; β-actin(promoter), 5′- TGCACTGTGCGGCGAAGC-3′ and 5′-TCGAGCCATAAAAGGCAA-3′. Each ChIP assay was performed at least twice to ensure reproducibility, and the linearity of the semi-quantitative PCR analysis was routinely measured for the different genes.

2.6. Statistical analysis

Statistical significance was estimated with the Student's two-tailed *t*-test for unpaired observations. A P value of less than 0.05 was considered to be significant.

3. Results

3.1. Functional analysis of the 5′ flanking region of the human SREBP-1a promoter

We investigated the regulation of the human SREBP-1a promoter by generating a series of progressive 5′ deletion reporter gene constructs, that extended over 2 kb of the ATG translation start site (expressed as +1), and we then proceeded to transfect these constructs into HEK293T cells. Firstly, we confirmed that this cell line expressed the expected SREBP-1 proteins with a majority SREBP-1a transcript expression (Fig. 1A). Analysis of reporter constructs revealed that a region at 75 bp upstream of the ATG start codon was sufficient to retain full promoter activity (Fig. 1B). Fig. 2A shows the human SREBP-1a promoter region between nucleotides −310 and +285, along with some putative binding sites for transcriptional regulators which were predicted by TRANSFAC database analysis. This promoter sequence did not contain a TATA box while various GC boxes were identified, which could be potential binding sites for SP1 family members. In order to verify the contribution of the GC-boxes to the overall activation, we generated single, double or triple mutant reporter plasmids (Fig. 2B). Upon transient transfection into HEK293T cells, a dramatic drop in the reporter activity was observed in the single mutant, which we named C, or in the double or triple mutants, with a luciferase activity similar to the pGL3-basic vector. These data indicated a major, if not exclusive, transcriptional activation from the GC boxes. Furthermore, the 3′-site appears to be crucial for promoter activity in HEK293T cells. The computer analysis (TRANSFAC) of the human SREBP-1a promoter predicted two putative nuclear factor kappa B (NFκB) binding sites and a CCAAT box (a NF-Y binding site) immediately upstream of the GC boxes, that were not present in the mouse or rat sequences (Fig. 2A). However, a mutation of these putative elements did not reduce promoter activity significantly (Fig. 2C).

3.2. Identification of SREBP-1a activators

In order to evaluate whether the GC boxes of the −77 to −1 bp minimal promoter region bind SP1 transcription factors, three oligonucleotides (indicated as probes A, B or C in Fig. 3) were used in the EMSA assay in the presence of recombinant human SP1 protein. The three SP1 binding sites were well recognized by the SP1 factor since the complex was abolished in the presence of unlabelled oligonucleotides, and was partially supershifted with an anti-SP1 monoclonal antibody. Moreover, competition was not observed when SP1-mutated oligonucleotides were used (see lanes mA, mB and mC). These results demonstrate that the putative SP1 elements, essential for basal human SREBP-1a promoter activity, bind the SP1 transcription factor in vitro.

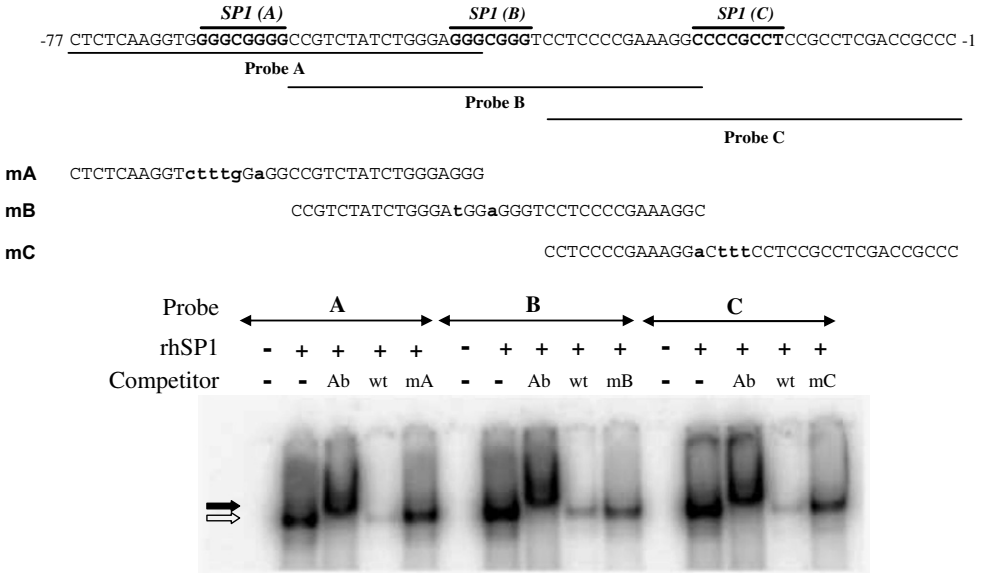

Fig. 3. SP1 transcription factor binds to the minimal human SREBP-1a promoter. An autoradiogram from a typical EMSA is shown in the bottom panel. The sequence of the human SREBP-1a core promoter and the sequences for individual probes used in the EMSA studies are shown. Mutations generated in the probes are noted in the lower case. The white arrow denotes SP1 binding signal, while the black arrow represents the supershifted SP1 binding signal.

3.3. Overlapping SP1/EGR-1 elements regulate the human SREBP-1 a promoter

We observed that the sequence between −77 and −1 bp contained overlapping binding sites for SP1 and EGR-1 (Fig. 4A). We examined competition for binding using oligonucleotides A, B and C described in Fig. 3. When DNA probes were mixed with either protein factor alone, single complexes of characteristic migration rates were detected (Fig. 4B). Both SP1 and EGR-1 bound separately and specifically to DNA fragments when presented together. An EGR-1 specific antibody removed only the lower retarded band corresponding to EGR-1 without disturbing SP1 binding. Then, we also tested whether EGR-1 could bind to this region by competing with SP1 to the three GC elements. When increasing amounts of EGR-1 were added with a constant amount of SP1, EGR-1 was preferentially bound to each GC element in the promoter to compete with SP1 at its binding sites (Fig. 4C).

To verify the in vivo binding of the transcription factors SP1 and EGR-1 in the SREBP-1a promoter, we carried out a ChIP assay in growing HEK293T cells. After immunoprecipitation and cross-link reversal, enrichment of the human SREBP-1a promoter was monitored by PCR amplification using specific primers for the promoter region −277 to +51 (Fig. 4D). Factors SP1 and EGR-1 were present in the human SREBP-1a promoter, whereas we were unable to precipitate the DNA fragments corresponding to the SREBP-1a coding region. In order to verify the results obtained, the same samples employed for the ChIP assay were used for the RNApol-ChIP

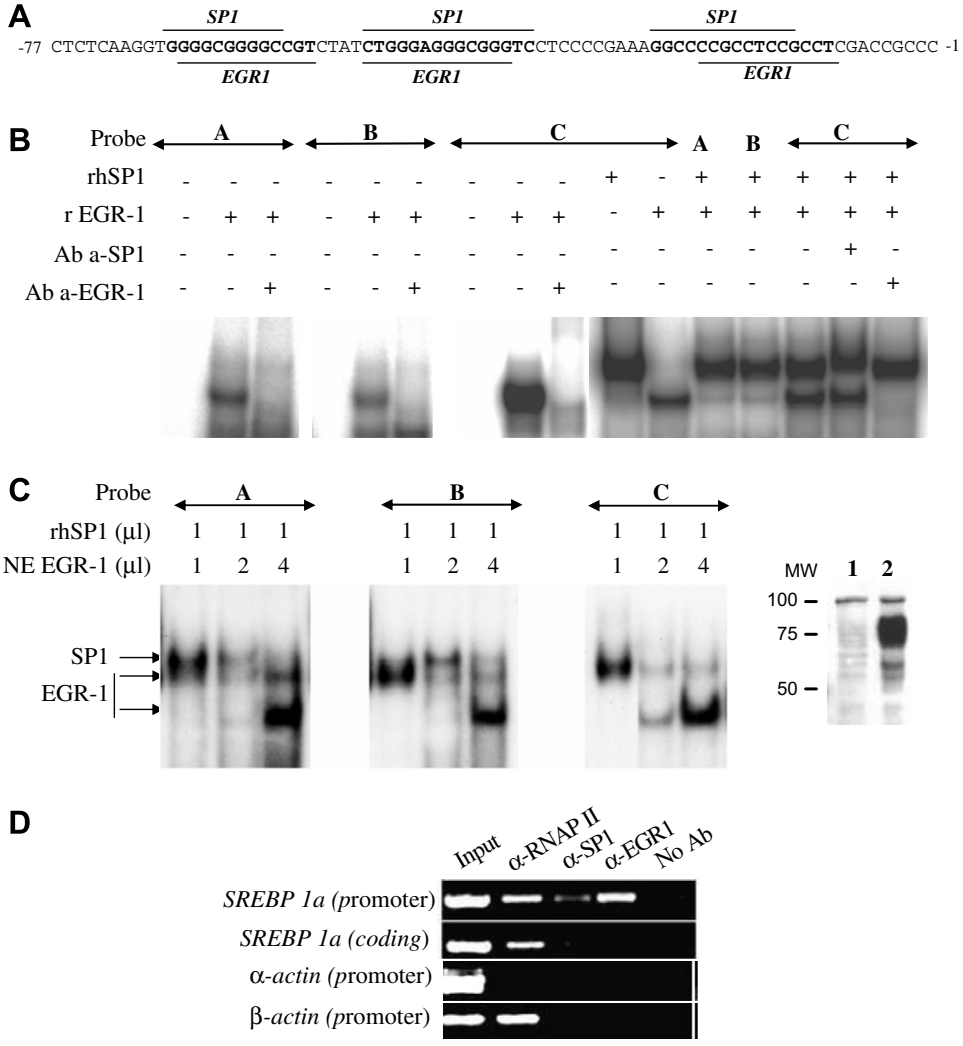

Fig. 4. SP1/EGR-1 binding to overlapping sites in the human SREBP-1a promoter. (A) The DNA sequence for the human SREBP-1a proximal-promoter, along with locations of the putative overlapping SP1/EGR-1 elements, is shown. (B) Recombinant SP1 and EGR-1 proteins were used in a standard gel shift analysis as described in Section 2. Labelled probes, whose sequences were described in Fig. 3, were incubated with EGR-1 alone or with both proteins together prior to electrophoresis, as indicated at the top of the figure. Identity of protein–DNA complexes was ensured using specific antibodies. (C) A representative autoradiogram of three independent experiments shows competition for SP1 binding by increasing levels of nuclear extracts, prepared from HEK293T cells transiently transfected with a pcDNA3-*egr1* expression vector. Western blot analysis of EGR-1 levels in nuclear extracts from cells transfected with native control (lane 1) or Egr-1 expression vector (lane 2) is shown. (D) Soluble chromatin was prepared from HEK293T cells. The PCR analysis of DNA extracted from input samples (input), the immunoprecipitates with the indicated antibodies, and of the samples treated without an antibody (*NoAb*), using amplicons from the promoter or coding regions of the indicated genes, is shown. A PCR analysis of the promoter regions of the α- and β-actin genes, as positive and negative controls of the ChIP and RNApol ChIP analysis, respectively, is also shown.

assay, as previously described [11]. The α-RNA polymerase antibody precipitated both promoter and coding chromatin fragments, thus confirming that the SREBP-1a transcript is expressed in HEK293T cells.

In the light of the results obtained, we evaluated whether EGR-1 directly affects the human SREBP-1a promoter activity. As Fig. 5A depicts, EGR-1 is able to inhibit the −75/+194luc SREBP-1a luciferase activity. The EGR-1 overexpression was able to increase the luciferase activity of the pEBS1^{4}luc reporter vector as a positive control. This plasmid encompasses four binding sites for EGR-1 derived from the *egr-1* promoter immediately upstream of the TATA box [5]. The essential role of SP-1 in the positive regulation of the human SREBP-1a promoter was also evaluated in HEK293T cells in transient transfection experiments (Fig. 5B). The expression of SP1 led to increased luciferase reporter activity under the control of the SREBP-1a promoter. A reporter gene, under the control of four copies of a GC-rich genetic element of the aldolase C gene, a TATA box, and the luciferase open reading frame (pALd-GCB^4), was used as an SP1-dependent luciferase expression vector [5].

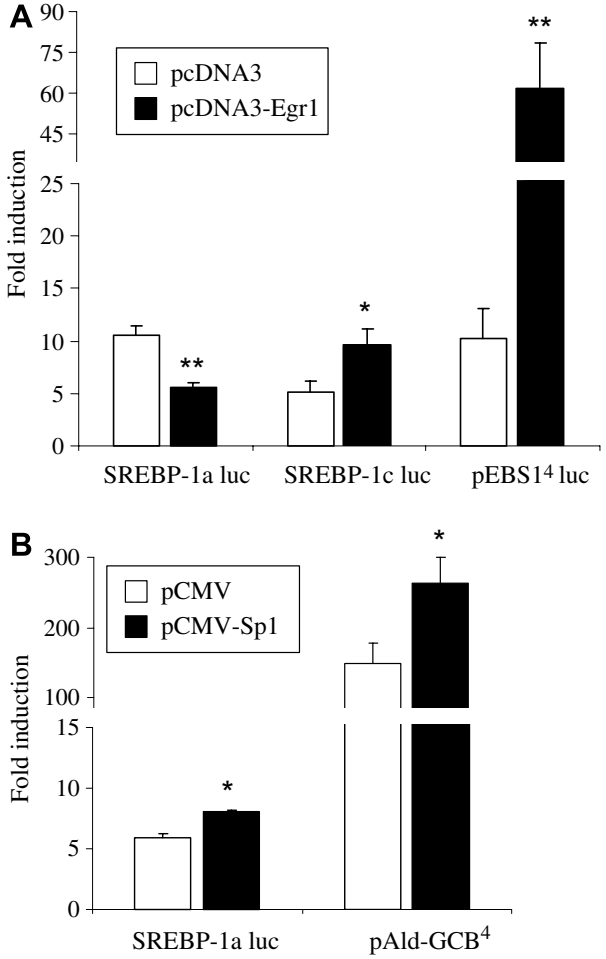

Fig. 5. EGR-1 overexpression represses the human SREBP-1a promoter. Reporter plasmids and the pcDNA-*egr1* (panel A) or pCMV-Sp1 (panel B) expression vector were transiently co-transfected into HEK293T cells. After 24 h, cells were lysed to measure luciferase activities. The EGR-1 data are represented as the mean firefly/renilla luciferase ratio relative to the activity of the empty expression vector (set at 100%). For the Sp1 analysis, the mean firefly luciferase/protein ratio was considered. Values represent the means ± S.E.M. of four transfections run in duplicate; $^{**}P < 0.01$; $^{*}P < 0.05$.

To confirm the inhibitor role of EGR-1, HEK293T cells were transfected with gene-specific siRNA. After transfection with siRNA, the mRNA expression of *egr-1* was specifically knocked down by 75% (Fig. 6B). In contrast, the promoter activity of the −75 bp fragment, or the endogenous SREBP-1 expression, was significantly higher than those obtained in cells transfected with the siRNA control (Fig. 6A and B). However, the activity of the EGR-1 positive pEBS1^{4}luc reporter vector diminished. The reciprocal effect was observed after specifically knocking down SP1.

4. Discussion

In this report, we have identified the core human SREBP-1a promoter region. The human promoter, like the mouse promoter [4], is enclosed in a small region of DNA that is characterized by a GC-rich region. Adamson et al. proposed three "levels of certainty" for the identification of EGR-1 target genes [12]: (i) a correlation of the *egr-1* expression with the indicated gene using *egr-1* inducible signal molecules or *egr-1* expression vectors (level 1); (ii) the in vitro identification of EGR-1 binding to the promoter of the gene (level 2) and (iii) the in vivo verification of EGR-1 binding by the chromatin immunoprecipitation assay (level 3). Having followed the three aforementioned levels (Figs. 4 and 5), our studies revealed that the EGR-1 and SP1 binding sites overlap within the SREBP-1a proximal promoter. These overlapping sites are present in other promoters, in most of which the binding of both transcription factors is mutually exclusive [6]. The data in Fig. 4 show that elevated amounts of EGR-1 can displace pre-bound SP1 from the GC elements. Moreover, the three GC boxes do not have the same EGR-1 binding capacity. EGR-1 binds more efficiently to probe C in comparison with probes A and B (Fig. 4C). This main role of the 3′ site was also described in the mouse core SREBP-1a promoter [4], thus emphasizing that this element is essential for the regulated expression of the SREBP-1a transcript.

There are well documented examples of the dual role of EGR-1 as either an activator or transcriptional repressor [13]. Our evidence suggests that EGR-1 functions as a transcriptional repressor for the human SREBP-1a promoter. This inhibitor role was specific for the SREBP-1a promoter because a forced expression of *egr-1* slightly increased the activity of the human SREBP-1c promoter (Fig. 5A).

Egr-1 is widely expressed and regulates a range of cellular processes, including proliferation, growth and apoptosis [14]. The proposed role of EGR-1 in controlling cell growth is based on the correlation between the mitogenic response and EGR-1 biosynthesis. However, the fact that the overexpression of *egr-1* neither induces cell death nor proliferation suggests that EGR-1 alone is insufficient to induce changes in physiological parameters [14]. Recently, Nakakuki et al. reported that SREBP-1a regulates cell growth [15]. The authors pointed out the biphasic effects of SREBP-1a, depending on its nuclear amount; at low expression levels, it promotes proliferation, whereas an overexpression of SREBP-1a blocks cell growth. The regulation of the SREBP-1a expression exercised by

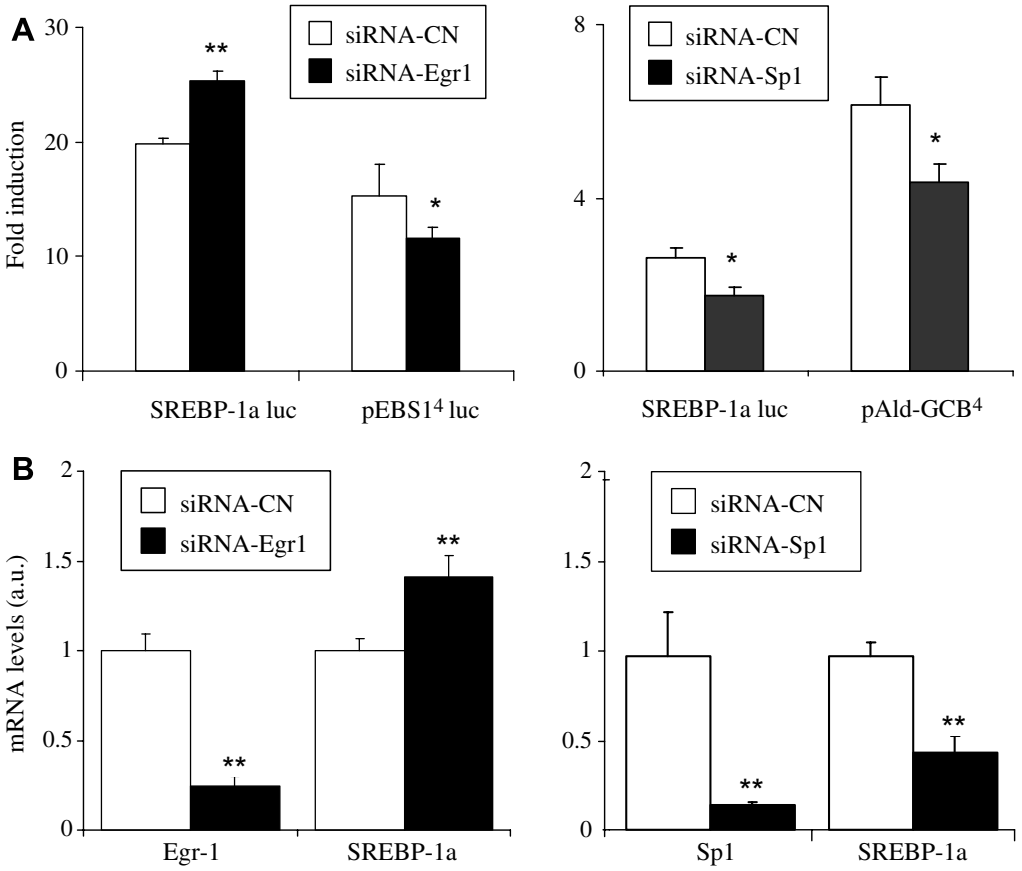

Fig. 6. Knockdown experiments confirm the reciprocal regulation of the human SREBP-1a by EGR-1 and Sp1. (A) HEK293T cells transfected with reporter plasmids and specific siRNAs. The fold induction relative to the pGL3-basic vector (±S.E.M.), normalized to renilla luciferase data, was calculated. The results shown are the average of four transfections run in duplicate; $^{**}P < 0.01$; $^{*}P < 0.05$. (B) The Egr-1, Sp1 or SREBP-1a expression, normalized to the GAPDH expression, on HEK293T cells after transfection with gene-specific siRNAs. Asterisks indicate a significant difference ($^{*}P < 0.05$; $^{**}P < 0.01$) compared with cells with the negative siRNA control used as a calibrator.

EGR-1, and proved in our study, would reveal new aspects to be taken into account in the control of cell cycle and growth.

In conclusion, we have performed a functional characterization of the human SREBP-1a promoter, and have mapped the basal promoter region required for transcriptional activation. Furthermore, we have pointed out the functional role of the three GC-boxes containing overlapping EGR-1/SP1 binding sites.

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